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An Enzyme-Immobilization Procedure for the Analysis of Enzyme-Inhibiting Chemicals in Water

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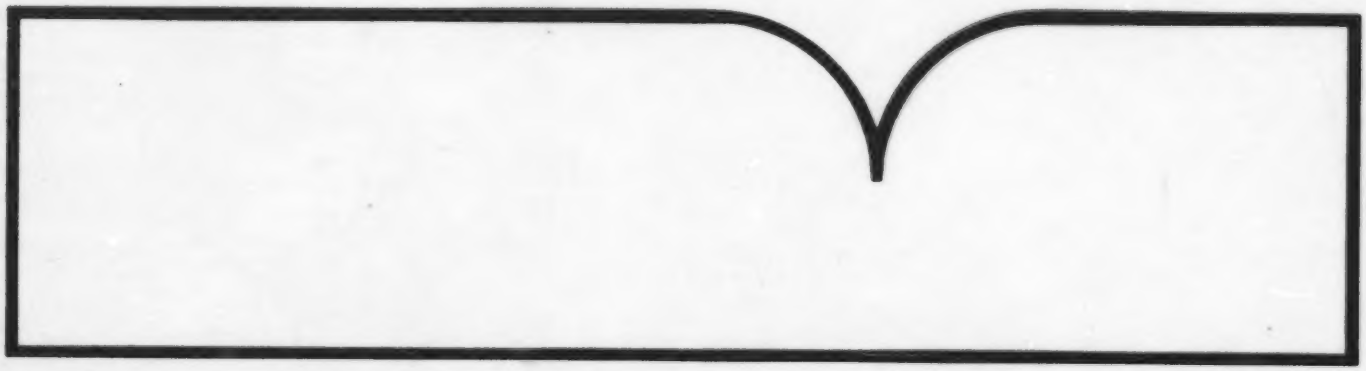

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16. ABSTRACT

The enzymes cholinesterase and urease wre mixed individually with gelatin and immobilized onto the inside surface of glass capillary tubes. After the gelatin-enzyme mixture had dried, water samples containing various enzyme inhibiting test chemicals were pumped through the tubes to enable interaction to proceed between the foreign agents and the enzymes. Substrate for each enzyme was later passed through the tubes and any difference in relative enzyme activity determined. This procedure may be useful for health and environmental studies, particularly in the rapid testing for micro- and nanomolar quantities of enzyme-inhibiting pollutants in natural waters and complex effluents.

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An Enzyme-Immobilization Procedure for the Analysis of Enzyme-Inhibiting Chemicals in Water

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The enzymes cholinesterase and urease were mixed individually with gelatin and immobilized onto the inside surface of glass capillary tubes. After the gelatin-enzyme mixture had dried, water samples containing various enzyme inhibiting test chemicals were pumped through the tubes to enable interaction to proceed between the foreign agents and the enzymes. Substrate for each enzyme was later passed through the tubes and any difference in relative enzyme activity determined. This procedure may be useful for health and environmental studies, particularly in the rapid testing for micro- and nanomolar quantities of enzyme-inhibiting pollutants in natural waters and complex effluents.

KEY WORDS: Enzyme, immobilization, cholinesterase, urease, monitor, water pollutant.

INTRODUCTION

Laboratory tests incorporating sensitive molecular indicators are needed to serve as rapid screening devices to signal the presence of toxic chemicals in environmental samples. Enzymes appear to be useful for this purpose, although the concept is still experimental and the limitations are largely unknown. Although the toxic mode of action of many classes of chemical pollutants on animals is largely conjectural, enzymes appear often to be primarily involved, as, for example, in the poisoning of higher animals by carbamate and organophosphate insecticides through acetylcholinesterase inhibition,[1] and by lead salts through delta-amino-levulinic acid dehydratase inhibition.[2] Some data suggest that many organochloride pesticides exert a toxic effect through adenosine-triphosphatase inhi-

bition;[3,4] and reagents carrying the sulfonamide group, as in acetazolamide, have a profound effect at very low concentrations on carbonic anhydrase activity.[5,6] There is some degree of correlation between enzyme-inhibition indices and lethality values for fish for a group of toxicants determined in previous studies,[7] which indicates that enzyme inhibition has some relevance to toxicity of the whole animal.

The fact that enzymes can be immobilized and thus insolubilized onto an inert support, while maintaining full activity, renders them especially useful for purposes as here discussed. Considerable information is available concerning the immobilization of enzymes, including for example, data on types of enzymes studied, kinds of binding agents tested, types of inert supports used, and various procedures for the analysis of activity.[8–11] Analyses involving immobilized enzymes have had some application concerning environmental contamination problems.[12–15]

The methodology described herein was developed with acetylcholinesterase (AChE; E.C.3.1.1.7) and urease (E.C.3.5.1.5.). When monitoring foreign chemicals in test water samples, it is expected that many organic compounds with the carbamate, phosphate, or thiophosphate functional group may impair AChE activity, although many other classes of chemicals also inhibit this enzyme, including heavy metal ions.[16] Likewise, although urease is known to be highly inhibited by heavy metal salts,[17] it is also affected by other compounds and therefore could serve as a biochemical monitor for a variety of classes of chemicals.[18,19]

MATERIALS AND METHODS

In parallel operations, three aqueous solutions of gelatin (0.18 g/l of 40° water) were mixed with 300 μl of either water (blank), urease solution (2.0 mg/ml), or AChE solution (4.0 mg/ml).†‡ The viscous preparations were then drawn up to the reference mark of each of as many as 26 capillary tubes of 100 μl capacity and allowed to drain.§ The tubes were then connected to flexible tubing (0.63 mm i.d.), were positioned in a peristaltic pump‖ (Figure 1), and air pumped (7 ml) through the system for 30 min to promote drying. Deionized water was then pumped through the

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†Sigma Chemical Co., P.O. Box 14508, St. Louis, MO, 63178, U.S.A.: Urease-Type IX—Jack Bean; Acetylcholinesterase—Type I—Bovine; Gelatin—Type I—Swine skin.

‡Mention of commercial products does not imply endorsement by the U.S. EPA.

§Accu-Fill "90"—pipettes—100 μl—Clay Adams, Becton Dickinson & Co., Parsippany, NY, 07054, U.S.A.

‖Autoanalyzer Proportioning Pump—II; Technicon Instrument Co., Tarrytown, NY, 10591, U.S.A.

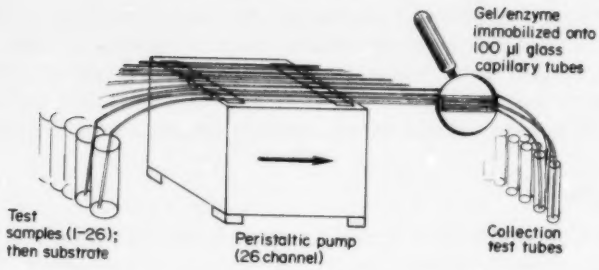

FIGURE 1 Instrumentation arrangement for the analysis of liquid samples of water pollutants, showing urease and acetylcholinesterase immobilized onto the interior of glass capillary tubes.

tubes for 30 min (7 ml) to wash unreacted materials from the immobilized matrix. Polluted water samples or water samples containing various concentrations of test chemicals, along with control water samples, were then pumped "across" the enzyme matrix for a unit of time (15 to 240 min, 3.5 to 55 ml) depending upon the reactivity and concentration of the water-borne chemicals. The enzyme substrates, 4.0 mM acetylthiocholine iodide† in 20 mM phosphate buffer at pH 7.0 for cholinesterase and 0.10 M urea† in 1.0 mM phosphate buffer at pH 6.0 for urease, were then pumped through the system. After 4 min, the first increment (0.9 ml) was discarded and pumping was continued for 14 min, whereby 3.2 ml samples were collected for analysis and the products then determined according to conventional methods. Urease activity was found by measuring the pH change caused by the liberation of ammonia from the hydrolysis of urea.[20] The concentration of substrate and pH of the buffer were such that an enzyme-free preparation provided a pH of 6.00 (minimum value), and the control enzyme preparation (no inhibition) produced ammonia to generate a pH of 9.00 (maximum value). The percentage of activity for the enzyme samples exposed to inhibitors was determined within the range of 6.00 to 9.00. Cholinesterase was analysed according to the colorimetric method of Ellman,[21] whereby the collected samples were treated with 5,5′dithiobis-2-nitrobenzoic acid (1.0 ml of 2.0 mM), and the absorbance determined at 418 nm with glutathione as the standard. Enzyme-free preparations provided blank absorbance values, and control enzyme preparations (no inhibition) provided values showing maximum activity (about 0.8 Abs.).

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†Sigma Chemical Co., P.O. Box 14508, St. Louis, MO, 63178, U.S.A.: Acetylthiocholine iodine; Urea.

The percentage of activity from the enzyme samples exposed to inhibitors was determined within the absorbance range of these limits. The analyses of water samples or test reagents were carried out in duplicate and usually were composed of a set of controls (no toxicant), and toxicants or pollutants in water samples of low, medium, and high concentration.

RESULTS

The limit of sensitivity of these two enzymes was found for two different types of chemical toxicants, cupric chloride and the carbamate, carbaryl.† The threshold (effect–no effect) concentration for carbaryl was 10^{-7} M (20.1 ppb) after 1 hr of irrigation (13.8 ml) and 10^{-8} M (2.01 ppb) after 4 hr (55.2 ml). Urease was unaffected at concentrations up to 10^{-4} (20.1 ppm) after 4 hr. Cupric chloride ($CuCl_2 \cdot 2H_2O$) caused a measurable effect upon AChE at 10^{-6} M (171 ppb) after 1 hr and at 10^{-8} M (1.71 ppb) after 4 hr; urease was inhibited at 10^{-7} M (17.1 ppb) after 1 hr and at 10^{-9} M (0.171 ppb) after 4 hr.

The thermal stability of the two enzymes immobilized onto glass capillaries was determined. At room temperature the half-life activity was about 2 days for cholinesterase and 5 days for urease; at 1°C the half-life was 3 days for cholinesterase and 10 days for urease; and at −30°C the values were estimated to be about 1 month for cholinesterase and 4 months for urease. The tenacity of adsorption of the matrix to the glass was determined by continuously pumping water through the tubes. For AChE, after 4 hr (276 ml) the loss of activity was 30%, and after 8 hr (552 ml) it was 50%. For urease, after 4 hr the loss was 5%, and after 8 hr it was 9%. The binding agents, collagen, gum arabic, gum damar, gum accroides, gum guaiac, gum xanthan, agar, and agarose, in aqueous solution were tested in place of gelatin but found to be inferior. Glutaraldehyde was tested as a potential stabilizer of the gelatin-enzyme preparation, but found not to improve the analysis.

A number of preliminary screening experiments were carried out with miscellaneous water samples readily available and with some water samples containing environmental pollutants from on-going fish bioassay tests at this laboratory. For example, analyses were carried out and enzyme-inhibition effects observed, with dilute (ppb range) aqueous solutions of mercuric chloride, cadmium chloride, dicofol, chloropyrifos, and pydrin.‡ Further water analyses, including field tests, are underway.

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†Carbaryl = seven, 1-napthal-N-methylcarbamate (insecticide).

‡Dicofol = kelthane; 4,4′dichloro-α-[trichloromethyl]benzhydrol (insecticide); Pydrin = α-cyano-3-phenoxybenzyl-4-chloro-α-(1-methylethyl) phenylacetate (pesticide); Chloropyrifos = dursban; 0,0-diethyl-0-(3,5,6-trichloro-2-pyridinyl) phosphorothioate (insecticide).

DISCUSSION

The method here described was developed in order to screen relatively large numbers of water samples and detect nanomolar quantitites of potentially biocidal pollutants using as simple and as sensitive a procedure as possible. For the analysis of toxicants in on-going tests with water samples, for example, the enzyme capillary tubes can be prepared en masse, and then stored in the frozen state and used as needed. The tests are relatively simple to prepare, are inexpensive, can be adjusted so as to increase or decrease the sensitivity of a test, and involve instrumentation and equipment that could be used at a remote site. Further work is underway in testing other enzymes for incorporation into this system, which may show a greater sensitivity than AChE and urease to certain classes of environmental contaminants.

The practical usefulness and limits of biochemical tests for antipollution studies would be more fully developed through further toxicobiochemical testing *in vitro* and *in vivo* with fish and other experimental animals. If the toxic mode of action was known for each major chemical class of poisoning agent, so that the primary target sites of invading chemicals were identified and the initial perturbations understood, it would be possible to choose the optimum biochemical indicators of toxic pollution.

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Note added in proof: There are two U.S. Government publications concerning the use of enzymes as water monitors: EPA-R2-72-010 (Aug. 1972) and EPA-600/2-80-083 (May 1980); U.S. Environmental Protection Agency, Washington, D.C., U.S.A. 20460.